Measuring Adeno-Associated Virus (AAV) Vector Genome Titer Using Droplet Digital PCR Protocol

Protocol

Droplet Digital PCR

Bulletin 7407

Abstract

Exact viral titration of vector genome copies is critical for ensuring accurate and reproducible dosing in both preclinical and clinical settings. It is equally important for optimal transduction efficiency and downstream potency of cell therapies. Droplet Digital PCR (ddPCR) can directly quantify DNA copies with exemplary precision without a standard curve. AAV vector genome titer measurement with Droplet Digital PCR offers a significantly lower coefficient of variation (<10% CV) compared to quantitative PCR (qPCR) across both in-process and purified samples. This results in consistency across all manufacturing stages (Dobnik et al. 2019). This optimized protocol is designed as a starting point for the measurement of AAV vector genome titer and it is recommended that the end user validate their assay with this protocol prior to use.

Overview

This protocol is designed as a starting point for the measurement of AAV vector genome titer. A list of instruments and materials, reagent preparation steps, and methods are provided. Example results from an AAV vector genome titer measurement of a single-stranded DNA viral sample using an ITR2/eGFP Expert Design Duplex Assay is also documented. It is recommended that the end user validate their assay with this protocol prior to use. See Figure 1 for a schematic of the AAV assay workflow for Droplet Digital PCR.

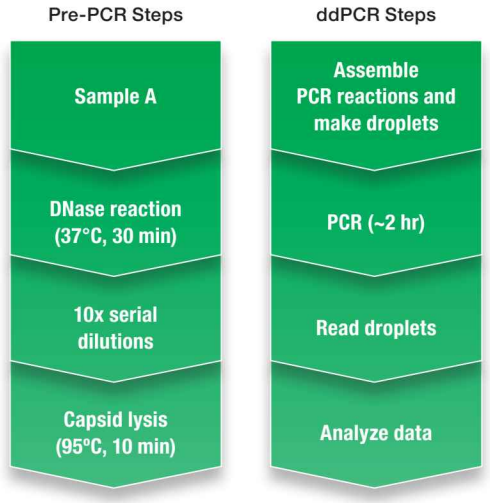

Fig. 1. Overview of the AAV assay workflow.

Instruments and Materials

Instruments

- QX200 Droplet Generator (Bio-Rad, catalog #1864002)
- QX200 Droplet Reader (Bio-Rad, #1864003)
- QX200 AutoDG Droplet Digital PCR System (Bio-Rad, #1864100)
- PX1 PCR Plate Sealer (Bio-Rad, #1814000)
- C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (Bio-Rad, #1851197)

Materials

- Poly(A), Polyadenylic Acid (Sigma-Aldrich Corporation, 10108626001)
- DNA Suspension Buffer, DNase/RNase Tested, PCR Grade (10 mM Tris, pH 8.0, 0.1 mM EDTA; Teknova Inc., T0223)
- Nuclease-Free Water (not DEPC-Treated) (Thermo Fisher Scientific Inc., AM9937)
- Pluronic F-68 Non-Ionic Surfactant (Thermo Fisher Scientific, 24040032)
- Eppendorf DNA LoBind Microcentrifuge Tubes (Fisher Scientific, 13-698-791)
- DNase I (RNase-free) (10x DNase I Reaction Buffer is included with the DNase I; New England Biolabs [NEB] Ltd., M0303)
- Restriction enzyme: MspI (New England Biolabs, R0106S)
- 0.2 ml 8-Tube PCR Strips and Domed Cap Strips (Bio-Rad, #TBC0802)
- Optical Flat 8-Cap Strips for PCR Tubes (Bio-Rad, #TCS0803)

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- DG8 Cartridges for QX200 Droplet Generator (Bio-Rad, #1864008)
- Droplet Generation Oil for Probes (Bio-Rad, #1863005)
- Automated Droplet Generation Oil for Probes (Bio-Rad, #1864110)
- ddPCR Supermix for Probes (No dUTP) (Bio-Rad, #1863023)
- PCR Plate Heat Seal (foil seal; Bio-Rad, #1814040)

Note: Additional materials and equipment are needed to carry out the Droplet Digital PCR portions of this protocol.

AAV Assay Workflow for Droplet Digital PCR

Reagent Preparation

1. Prepare a poly(A)+ solution using DNA Suspension Buffer (10 mM Tris-HCl, pH 8, 0.1 mM EDTA, 100 µg/ml poly[A], 0.01% Pluronic F-68).
   - Make a 50 mg/ml poly(A) stock solution by adding 2 ml of DNA Suspension Buffer to the vial of poly(A). Extensive vortexing is necessary. Store the poly(A) stock solution at 4°C
   - For 250 ml of DNA Suspension Buffer, combine 0.5 ml of 50 mg/ml poly(A) stock solution and 250 µl of stock 10% Pluronic F-68. Mix well. Store this poly(A)+ solution at 4°C when not in use
2. Prepare 10x Pluronic F-68 (1% solution) for DNase I digestion. Use a freshly prepared 1:10 dilution of the stock 10% solution in nuclease-free water. For small volumes, 10 µl of stock Pluronic F-68 is added to 90 µl of nuclease-free water in a DNA LoBind Microcentrifuge Tube.

Methods

Step 1: Digest Unencapsidated DNA Using DNase I

Choose an appropriate reaction setup, depending on the number of viral titer samples to be tested. Two possible reaction setups are shown in Table 1.

Table 1. Reaction setups for digestion.

Component	Volume/Reaction, µl	
10x DNase I Reaction Buffer	5	6
10x Pluronic F-68 Solution	5	6
DNase I (2 U/µl)	5	6
AAV sample	5	3
Nuclease-Free Water (not DEPC-Treated)	30	39
Total volume	**50**	**60**

1. Prepare a master mix containing all the components except the virus.
   - Multiply the volumes by the required number of reactions plus 1 extra reaction
   - Add water followed by 10x reaction buffer, 10x Pluronic F-68, and DNase to a DNA LoBind Microcentrifuge Tube. Pulse mix, briefly microcentrifuge, and then aliquot 45 µl (50 µl reaction) or 57 µl (60 µl reaction) into individual tubes of 0.2 ml 8-Tube PCR Strips
   - Add an appropriate volume of virus and cap the 8-Tube PCR Strips with Optical Flat 8-Cap Strips. Flick several times to mix, then microcentrifuge to bring the solution to the bottom of the tubes
2. Incubate in a C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module for 30 min at 37°C, then cool to 4°C at 3°C/sec.

Step 2: Dilute AAV Samples to ddPCR Detection Range Using Poly(A)+ Solution

For initial experiments on purified AAV of unknown concentration, we recommend preparing six tenfold serial dilutions of the virus after DNase digestion in DNA LoBind Microcentrifuge Tubes for testing. Suggestions for the tenfold dilutions are in the example section of the protocol.

Step 3: Lyse Capsid

1. For the last 4 dilutions, aliquot 20 µl of each dilution into 0.2 ml 8-Tube PCR Strips and cap with Optical Flat 8-Cap Strips.
2. Pulse the tubes in a microcentrifuge to ensure that the solution is at the bottom of the tubes.
3. Lyse the capsid for 10 min at 95°C, then cool to 4°C at 3°C/sec using a thermal cycler.
4. Thoroughly mix the samples by flicking the tubes after removing them from the PCR instrument, then microcentrifuge to bring the liquid to the bottom of the tubes.

Step 4: Prepare ddPCR Reactions

Choose an appropriate duplex ddPCR reaction setup, depending on the number of viral titer samples to be tested. Two possible ddPCR reaction setups are shown in Table 2.

Table 2. Duplex ddPCR reaction setups.

Component	1:20 DNA Dilution Volume/Reaction, µl	1:4 DNA Dilution Volume/Reaction, µl
ddPCR Supermix for Probes (No dUTP)	10	10
20x FAM assay (ITR2)	1	1
20x HEX assay (gene of interest)*	1	1
AAV DNA from step 3 after capsid lysis	1	5
5 U MspI (20,000 U/ml)	0.25	0.25
Nuclease-Free Water (not DEPC-Treated)	6.75	2.75
Total volume	**20**	**20**

* Use an enhanced green fluorescent protein (eGFP) assay for control virus.

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1. Prepare a master mix containing all the components except the DNA. Also see the Preparing a ddPCR Master Mix for Larger Volumes section.
   - Make 3 reactions for each viral DNA dilution: 2 technical replicates plus 1 extra reaction
   - Include 10% extra reactions for liquid loss to tube and pipet tip surfaces
   - Round the number of reactions up to a multiple of 4 so that the pipet volumes are integers
2. Pipet 57 µl (1:20 DNA dilution) or 45 µl (1:4 DNA dilution) of master mix into labeled DNA LoBind Microcentrifuge Tubes.
3. Pipet 3 µl (1:20 DNA dilution) or 15 µl (1:4 DNA dilution) of viral DNA into the appropriate tubes, then mix by vortexing.

Preparing a ddPCR Master Mix for Larger Volumes

Depending on the number of reactions, assembling and aliquoting a ddPCR master mix containing all the components, except the template DNA, can facilitate reaction preparation (see Table 3).

1. Adjust the number of reactions to include extra volume for liquid loss and any necessary replicates.

Table 3. ddPCR master mix setup.

Component	1 µl DNA Reaction Volume, µl	5 µl DNA Reaction Volume, µl
ddPCR Supermix for Probes (No dUTP)	10 x *n*	10 x *n*
20x FAM assay	1 x *n*	1 x *n*
20x HEX assay	1 x *n*	1 x *n*
Template DNA	–	–
5 U MspI (20,000 U/ml)	0.25 x *n*	0.25 x *n*
Nuclease-Free Water (not DEPC-Treated)	6.75 x *n*	2.75 x *n*
Total volume	**19 x *n***	**15 x *n***

n, number of reactions.

2. Add water followed by supermix, 20x assays, and MspI to a DNA LoBind Microcentrifuge Tube. Mix by vortexing, microcentrifuge, then aliquot for DNA addition.

Step 5: Generate Droplets and Seal Plate

For the manual QX200 Droplet Generator:

- Pipet 20 µl into the sample wells on the cartridge followed by 70 µl of Droplet Generation Oil for Probes into the oil wells
- Generate droplets, transfer them to a PCR plate, and seal with foil using a PX1 PCR Plate Sealer

For the automated droplet generator (QX200 AutoDG ddPCR System):

- Add 25 µl to each well on the plate. The extra volume is required to account for liquid loss to the surface of tubes and pipet tips
- Generate droplets and seal with foil using a PX1 PCR Plate Sealer

Step 6: Perform PCR Amplification

Use the thermal cycling protocol in Table 4 for PCR amplification.

Table 4. Thermal cycling protocol.*

Cycling Step	Temperature, °C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	10 min	2°C/sec	1
Denaturation	94	30 sec		40
Annealing/ extension	55	1 min		
Enzyme deactivation	98	10 min		1
Hold	4	Infinite		1

* Use a heated lid set to 105°C and a sample volume of 40 µl.

Step 7: Read Droplets

Set up a plate layout in QuantaSoft (version 1.7), QuantaSoft Analysis Pro, or QX Manager Software for the samples. Read the droplet fluorescence using a QX200 Droplet Reader.

Step 8: Analyze Data

Analyze the data and determine the concentration (copies/µl) for the ddPCR reactions using QuantaSoft (version 1.7), QuantaSoft Analysis Pro, or QX Manager Software. Use the appropriate dilution factor for each sample to backcalculate the titer of the initial solution.

Example: One Single-Stranded DNA Viral Sample with Two DNase I Replicates Using an ITR2/eGFP Duplex Assay

Step 1: DNase Digestion for 5 µl of Viral Sample

For two DNase replicates prepare a 3x master mix (for 2 replicates + 1 extra) according to the instructions in Table 5.

Table 5. Preparation of master mix.

Component	Volume/Reaction, µl	Master Mix, µl
10x DNase I Reaction Buffer	5	15
10x Pluronic F-68 Solution	5	15
DNase I (2 U/µl)	5	15
AAV sample	5	–
Nuclease-Free Water (not DEPC-Treated)	30	90
Total volume	**50**	**135**

1. Aliquot 45 µl of the master mix into 2 tubes of a 0.2 ml 8-Tube PCR Strip after the master mix is thoroughly mixed.
2. Add 5 µl of the virus to each tube containing master mix, cap tubes with an Optical Flat 8-Cap Strip, and mix by flicking the tubes. Briefly microcentrifuge the tubes.
3. Incubate in a C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module for 30 min at 37°C then cool to 4°C at 3°C/sec.

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Step 2: Dilute AAV Samples

Prepare serial tenfold dilutions of each replicate DNase reaction by aliquoting 180 µl of poly(A)+ solution into labeled DNA LoBind Microcentrifuge Tubes. Thoroughly vortex mix and pulse microcentrifuge each dilution before proceeding to the next dilution.

- DNase replicate 1:
  - Dilution 2: 20 µl DNase reaction + 180 µl poly(A)+
  - Dilution 3: 20 µl dilution 2 + 180 µl poly(A)+
  - Dilution 4: 20 µl dilution 3 + 180 µl poly(A)+
  - Dilution 5: 20 µl dilution 4 + 180 µl poly(A)+
  - Dilution 6: 20 µl dilution 5 + 180 µl poly(A)+
  - Dilution 7: 20 µl dilution 6 + 180 µl poly(A)+
- For DNase replicate 2, follow the DNase replicate 1 dilution scheme

Step 3: Lyse Capsid

1. For each replicate, aliquot 20 µl of the appropriate dilutions into 0.2 ml 8-Tube PCR Strips. Use dilution 4, dilution 5, dilution 6, and dilution 7 from Step 2: Dilute AAV Samples for each replicate.
2. Lyse the capsid for 10 min at 95°C, then cool to 4°C at 3°C/sec using a C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module.
3. Thoroughly mix the samples after removing them from the PCR instrument by flicking the tubes and then pulsing in a microcentrifuge to bring the samples to the bottom of the tubes.

Step 4: Prepare ddPCR Reactions

Follow the guidelines in Table 6 to make the master mix.

(2 DNase replicates) x (4 dilutions) =
8 samples x (2 technical replicates + 1 extra)
= 24 reactions

so, make a master mix for 28 reactions to make the volumes integer values and to account for liquid loss to surfaces.

Table 6. Preparation of master mix.

Component	Volume/Reaction, µl
ddPCR Supermix for Probes (No dUTP)	10 x 28 = 280
20x FAM assay (ITR2)	1 x 28 = 28
20x HEX assay (eGFP)	1 x 28 = 28
Template DNA	–
5 U MspI (20,000 U/m)	0.25 x 28 = 7
Nuclease-Free Water (not DEPC-Treated)	6.75 x 28 = 189
Total volume	**19 x 28 = 532**

1. Aliquot 57 µl of the master mix into 8 separate DNA LoBind Microcentrifuge Tubes (2 DNase replicates) × (4 dilutions) = 8 reactions.
2. Aliquot 3 µl of each DNA sample into the correspondingly labeled DNA LoBind Microcentrifuge Tube containing ddPCR master mix and mix by pipetting.
3. Pulse vortex each tube, then spin down in a microcentrifuge.

Step 5: Perform PCR Amplification

The ddPCR reactions can be loaded in one of these ways:

- Manually into a Droplet Generation Cartridge:
  - Add 20 µl from each tube into 2 separate sample wells of a DG8 Cartridge
  - Repeat until all 8 sample wells are filled
  - Add 70 µl of Droplet Generation Oil, using either a single-channel or multichannel pipet, into the oil wells of the DG8 Cartridge
- Manually into a PCR plate and then transferred into a Droplet Generation Cartridge:
  - Add 25 µl from each tube into 2 separate wells of a PCR plate
  - Pulse centrifuge to bring the samples to the bottom of the wells
  - Use a multichannel pipet to transfer 20 µl from each well into the sample wells of a DG8 Cartridge
  - Add 70 µl of Droplet Generation Oil, using either a single-channel or multichannel pipet, into the oil wells of the DG8 Cartridge
- Into a PCR plate for automated droplet generation:
  - Follow the instructions for the Automated Droplet Generator (QX200 AutoDG ddPCR System)

Generate droplets, transfer them to a PCR plate when using a manual droplet generator or remove droplet plate from Automated Droplet Generator (QX200 AutoDG ddPCR System), and seal with foil using a PX1 PCR Plate Sealer. An example plate layout is shown in Figure 2. The two technical replicate wells are the same color.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DNase replicate 1 Dilution 4	DNase replicate 2 Dilution 4										
B	DNase replicate 1 Dilution 4	DNase replicate 2 Dilution 4										
C	DNase replicate 1 Dilution 5	DNase replicate 2 Dilution 5										
D	DNase replicate 1 Dilution 5	DNase replicate 2 Dilution 5										
E	DNase replicate 1 Dilution 6	DNase replicate 2 Dilution 6										
F	DNase replicate 1 Dilution 6	DNase replicate 2 Dilution 6										
G	DNase replicate 1 Dilution 7	DNase replicate 2 Dilution 7										
H	DNase replicate 1 Dilution 7	DNase replicate 2 Dilution 7										

Fig. 2. Example of a plate layout.

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Step 6: Read Droplets

Set up a plate layout in QuantaSoft (version 1.7), QuantaSoft Analysis Pro, or QX Manager Software for the samples. Read the droplet fluorescence using a QX200 Droplet Reader. Sample data are shown in Figure 3.

Step 7: Analyze Data

Assume dilution 6 had a concentration of 2,500 copies/µl for eGFP. Dilution 7 should have ~250 copies/µl, so this data could be combined with the concentration from dilution 6 when calculating the average titer.

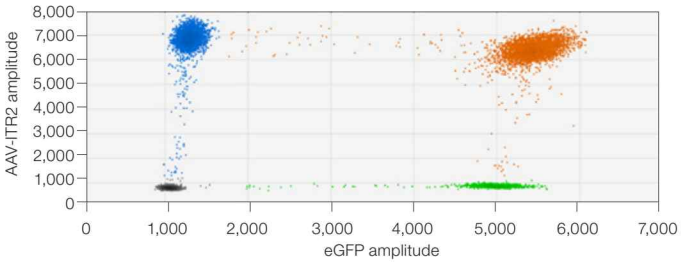

Fig. 3. Duplex ddPCR data of a recombinant AAV2-CMV-GFP viral sample (Vigene Biosciences, CV10004). AAV-ITR2–positive droplets (■); eGFP-positive droplets (■); double-positive droplets (■); and negative droplets (■).

Step 8: Calculate the Viral Titer using the eGFP Concentration

In general, the total dilution factor is:
Total dilution factor = (DNase dilution) x (dilution 2) x…x (dilution *n*) x (PCR dilution)

- For this example, the total dilution factor for dilution 6 is:

  Total dilution factor = (DNase dilution) x (dilution 2) x….x (dilution 6) x (PCR dilution)

  = (10)(10)(10)(10)(10)(10)(20)

  $= 2 \times 10^7$

- The viral titer, measured in vector genomes (VG), is:

  VG/µl = (2,500 copies/µl) x (2×10^7) = 5×10^{10}
  so,
  VG/ml = (1,000 µl/ml) x (VG/µl) = 5×10^{13}

Reference

Dobnik D et al. (2019). Accurate quantification and characterization of adeno-associated viral vectors. Front Microbiol 10, 1,570.

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